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(71)(72) Applicants and Inventors: KELLER, Gregory, S. [US/US]; Suite 105, 2323 De La Vina, Santa Barbara, CA 93105 (US). KLEINSEK, Don, A. [US/US]; W5036N County Highway A, Elkhart Lake, WI 53020 (US).

(74) Agents: WISE, Michael, J. et al.; Lyon & Lyon LLP, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).

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(54) Title: AUGMENTATION AND REPAIR OF DERMAL, SUBCUTANEOUS, AND VOCAL CORD TISSUE DEFECTS

(57) Abstract

The application concerns a method for corrective surgery of defects being amenable to rectification by the augmentation of tissue subjacent to the defect. The method involves retrieving viable cells from a subject, a neonate or human fetus. The cells are then cultured *in vitro* and placed into a tissue of the subject, the tissue being located in a position subjacent to the defect to be rectified. Alternatively, the cells may be cultured in a collagen matrix or suspended in a collagen matrix prior to being placed in a position subjacent to the defect to be rectified. In a further embodiment, the cells are cultured and the *in vitro* produced extracellular matrix is collected and placed in a position subjacent to the defect to be rectified. A method for the correction of vocal cord defects is also disclosed.

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SPECIFICATION

AUGMENTATION AND REPAIR OF DERMAL, SUBCUTANEOUS, AND VOCAL CORD TISSUE DEFECTS

This application claims the benefit of U.S. Provisional Application Serial No. 60/037,961 filed February 20, 1997.

FIELD OF INVENTION

The field of the present invention is the long-term augmentation and/or repair of dermal, subcutaneous, or vocal cord tissue.

BACKGROUND OF INVENTION

I. *IN VITRO* CELL CULTURE

The majority of *in vitro* vertebrate cell cultures are grown as monolayers on an artificial substrate which is continuously bathed in a nutrient medium. The nature of the substrate on which the monolayers may be grown may be either a solid (e.g., plastic) or a semi-solid (e.g., collagen or agar). Currently, disposable plastics have become a preferred substrate for cell culture.

While the growth of cells in two-dimensions is frequently used for the preparation and examination of cultured cells *in vitro,* it lacks the characteristics of intact, *in vivo* tissue which, for example, includes cell-cell and cell-matrix interactions. Therefore, in order to characterize these functional and morphological interactions, various investigators have examined the use of three-dimensional substrates in such forms as a collagen gel (Yang *et al.*, Cancer Res. 41:1027 (1981); Douglas *et al.*, In Vitro 16:306 (1980); Yang *et al.*, Proc. Nat'l Acad. Sci. 2088 (1980)), cellulose sponge (Leighton *et al.*, J. Nat'l Cancer Inst. 12:545 (1951)), collagen-coated cellulose sponge (Leighton *et al.*, Cancer Res. 28:286 (1968)), and GELFOAM® (Sorour *et al.*, J. Neurosurg. 43:742 (1975)). Typically, these aforementioned

three-dimensional substrates are inoculated with the cells to be cultured, which subsequently penetrate the substrate and establish a "tissue-like" histology similar to that found *in vivo*. Several attempts to regenerate "tissue-like" histology from dispersed monolayers of cells utilizing three-dimensional substrates have been reported. For example, three-dimensional collagen substrates have been utilized to culture a variety of cells including breast epithelium (Yang, Cancer Res. 41:1021 (1981)), vascular epithelium (Folkman *et al.*, Nature 288:551 (1980)), and hepatocytes (Sirica *et al.*, Cancer Res. 76:3259 (1980)), however long-term culture and proliferation of cells in such systems has not yet been achieved. Prior to the present invention, a three-dimensional substrate had not been utilized in the autologous *in vitro* culture of cells or tissues derived from the dermis, fascia, or lamina propria.

II. AUGMENTATION AND/OR REPAIR OF DERMAL AND SUBCUTANEOUS TISSUES

In the practice of cosmetic and reconstructive plastic surgery it is frequently necessary to employ the use of various injectable materials to augment and/or repair defects of the subcutaneous or dermal tissue, thus effecting an aesthetic result. Non-biological injectable materials (e g., paraffin) were first utilized to correct facial contour defects as early as the late nineteenth century. However, numerous complications and the generally unsatisfactory nature of long-term aesthetic results caused the procedure to be rapidly abandoned. More recently, the use of injectable silicone became prevalent in the 1960's for the correction of minor defects, although various inherent complications also limited the use of this substance. Complications associated with the utilization of injectable liquid silicone include local and systemic inflammatory reactions, formation of scar tissue around the silicone droplets, rampant and frequently-distant unpredictable migration throughout the

body, and localized tissue breakdown. Due to these potential complications, silicone is not currently approved for general clinical use. Although the original proponents of silicone injection have continued experimental programs utilizing specially manufactured "Medical Grade" silicone (e.g., Dow Corning MDX 4.4011®) with a limited number of subjects, it appears highly unlikely that its use will be generally adopted by the surgical community. See e.g., Spira and Rosen, Clin. Plastic Surgery 20:181 (1993); Matton *et al.*, Aesthetic Plastic Surgery 9:133 (1985).

It has also been suggested to compound extremely small particulate species in a lubricious material and inject such combination micro-particulate media subcutaneously for both soft and hard tissue augmentation and repair, however success has been heretofore limited. For example, bioreactive materials such as hydroxyapatite or cordal granules (osteo conductive) have been utilized for the repair of hard tissue defects. Subsequent undesirable micro-particulate media migration and serious granulomatous reactions frequently occur with the injection of this material. These undesirable effects are well-documented with the use of such materials as polytetrafluoroethylene (TEFLON®) spheres of small diameter (~90% of particles having a diameter of ≤ 30μm) in glycerin. See e.g., Malizia *et al.*, JAMA 251:3277 (1984). Additionally, the use of very small diameter particulate spheres (~1-20μm) or small elongated fibrils (~1-30μm in diameter) of various materials in a biocompatible fluid lubricant as injectable implant composition are disclosed in U.S. Patent No. 4,803,075. However. while these aforementioned materials create immediate augmentation and/or repair of defects, they also have a tendency to migrate and be reabsorbed from the original injection site.

The poor results initially obtained with the use of non-biological injectable materials prompted the use of various non-immunogenic, proteinaceous materials (e.g., bovine

collagen and fibrin matrices). Prior to human injection, however, the carboxyl- and amino-terminal peptides of bovine collagen must first be enzymatically-degraded, due to its highly immunogenic nature. Enzymatic degradation of bovine collagen yields a material (atelocollagen) which can be used in limited quantities in patients pre-screened to exclude those who are immunoreactive to this substance. The methodologies involved in the preparation and clinical utilization of atelocollagen are disclosed in U.S. Patent No. 3,949,073; U.S. Patent No. 4,424,208; and U.S. Patent No. 4,488,911. Atelocollagen has been marketed as ZYDERM® brand atelocollagen solution in concentrations of 35 mg/ml and 65 mg/ml. Although atelocollagen has been widely employed, the use of ZYDERM® has been associated with the development of anti-bovine antibodies in approximately 90% of patients and with overt immunologic complications in 1-3% of patients. See DeLustro *et al.*, Plastic and Reconstructive Surgery 79:581 (1987).

Injectable atelocollagen solution also was shown to be absorbed from the injection site, without replacement by host material, within a period of weeks to months. Clinical protocols calling for repeated injections of atelocollagen are, in practice, primarily limited by the development of immunogenic reactions to the bovine collagen. In order to mitigate these limitations, bovine atelocollagen was further processed by cross-linking with 0.25% glutaraldehyde, followed by filtration and mechanical shearing through fine mesh. The methodologies involved in the preparation and clinical utilization of this material are disclosed in U.S. Patent No. 4,582,640 and U.S. Patent No. 4,642,117. The modified atelocollagen was marketed as ZYPLAST® brand cross-linked bovine atelocollagen. The propertied advantages of cross-linking was to provide increased resistance to host degradation, however this was off-set by an increase in solution viscosity. In addition, cross-linking of the bovine

atelocollagen was found to decrease the number of host cells which infiltrated the injected collagen site. The increased viscosity, and in particular irregular increased viscosity resulting in "lumpiness," not only rendered the material more difficult to utilize, but also made it unsuitable for use in certain circumstances. See e.g., U.S. Patent No. 5,366,498. In addition, several investigators have reported that there is no or marginally-increased resistance to host degradation of ZYPLAST® cross-linked bovine atelocollagen in comparison to that of the non-cross-linked ZYDERM® atelocollagen and that the overall longevity of the injected material is, at best, only 4-6 months. See e.g., Ozgentas *et al.*, Ann. Plastic Surgery 33:171 (1994); and Matti and Nicolle, Aesthetic Plastic Surgery 14:227 (1990). Moreover, bovine atelocollagen cross-linked with glutaraldehyde may retain this agent as a high molecular weight polymer which is continuously hydrolyzed, thus facilitating the release of monomeric glutaraldehyde. The monomeric form of glutaraldehyde is detectable in body tissues for up to 6 weeks after the initial injection of the cross-linked atelocollagen. The cytotoxic effect of glutaraldehyde on *in vitro* fibroblast cultures is indicative of this substance not being an ideal cross-linking agent for a dermal equivalent which is eventually infiltrated by host cells and in which the bovine atelocollagen matrix is rapidly degraded, thus resulting in the release of monomeric glutaraldehyde 5into the bodily tissues and fluids.

Similarly, chondroitin-6-sulfate (GAG), which weakly binds to collagen at neutral pH, has also been utilized to chemically modify bovine protein for tissue graft implantation. See Hansborough and Boyce, JAMA 136:2125 (1989). However, like glutaraldehyde, GAG may be released into the tissue causing unforeseen long-term effects on human subjects. GAG has been reported to increase scar tissue formation in wounds, which is to be avoided in grafts. Additionally, a reduction of collagen blood clotting capacity

may also be deleterious in the application in bleeding wounds, as fibrin clot contributes to an adhesion of the graft to the surrounding tissue.

The limitations which are imposed by the immunogenicity of both modified and non-modified bovine atelocollagen have resulted in the isolation of human collagen from placenta (see e.g., U.S. Patent No. 5,002,071); from surgical specimens (see e.g., U.S. Patent No. 4,969,912 and U.S. Patent No. 5,332,802); and cadaver (see e.g., U.S. Patent No. 4,882,166). Moreover, processing of human-derived collagen by cross-linking and similar chemical modifications is also required, as human collagen is subject to analogous degradative processes as is bovine collagen. Human collagen for injection, derived from a sample of the patient's own tissue, is currently available and is marketed as AUTOLOGEN®. It should be noted, however, that there is no quantitative evidence which demonstrates that human collagen injection results in lower levels of implant degradation than that which is found with bovine collagen preparations. Furthermore, the utilization of autologous collagen preparation and injection is limited to those individuals who have previously undergone surgery, due to the fact that the initial culture from which the collagen is produced is derived is from the tissue removed during the surgical procedure. Therefore, it is evident that, although human collagen circumvents the potential for immunogenicity exhibited by bovine collagen, it fails to provide long-term therapeutic benefits and is limited to those patient who have undergone prior surgical procedures.

An additional injectable material currently in use as an alternative to atelocollagen augmentation of the subjacent dermis consists of a mixture of gelatin powder, ε-aminocapronic acid, and the patient's plasma marketed as FIBREL®. See Multicenter Clinical Trial, J. Am. Acad. Dermatology 16:1155 (1987). The action of FIBREL® appears to be dependent upon the initial induction of a sclerogenic

inflammatory response to the augmentation of the soft tissue via the subcutaneous injection of the material. See e.g., Gold, J. Dermatologic Surg. Oncology, 20:586 (1994). Clinical utilization of FIBREL® has been reported to often result in an overall lack of implant uniformity (i.e., "lumpiness") and longevity, as well as complaints of patient discomfort associated with its injection. See e.g., Millikan *et al.*, J. Dermatologic. Surg. Oncology, 17:223 (1991). Therefore, in conclusion, none of the currently utilized protein-based injectable materials appears to be totally satisfactory for the augmentation and/or repair of the subjacent dermis and soft tissue.

The various complications associated with the utilization of the aforementioned materials have prompted experimentation with the implantation (grafting) of viable, living tissue to facilitate augmentation and/or repair of the subjacent dermis and soft tissue. For example, surgical correction of various defects has been accomplished by initial removal and subsequent re-implantation of the excised adipose tissue either by injection (see e.g., Davies *et al.*, Arch. of Otolaryngology-Head and Neck Surgery 121:95 (1995); McKinney & Pandya, Aesthetic Plastic Surgery 18:383 (1994); and Lewis, Aesthetic Plastic Surgery 17:109 (1993)) or by the larger scale surgical-implantation (see e.g., Ersck, Plastic & Reconstructive Surgery 87:219 (1991)). To perform both of the aforementioned techniques a volume of adipose tissue equal or greater than is required for the subsequent augmentation or repair procedure must be removed from the patient. Thus, for large scale repair procedures (e.g., breast reconstruction) the amount of adipose tissue which can be surgically-excised from the patient may be limiting. In addition, other frequently encountered difficulties with the aforementioned methodologies include non-uniformity of the injectate, unpredictable longevity of the aesthetic effects, and a 4-6 week period of post-injection inflammation and swelling. In

contrast, in a preferred embodiment, the present invention utilizes the surgical engraftment of autologous adipocytes which have been cultured on a solid support typically derived from, but not limited to, collagen or isolated extracellular matrix. The culture may be established from a simple skin biopsy specimen and the amount of adipose tissue which can be subsequently cultured *in vitro* is not limited by the amount of adipose tissue initially excised from the patient.

Living skin equivalents have been examined as a methodology for the repair and/or replacement of human skin. Split thickness autographs, epidermal autographs (cultured autogenic keratinocytes), and epidermal allographs (cultured allogenic keratinocytes) have been used with a varying degree of success. However, unfortunately, these forms of treatment have all exhibited numerous disadvantages. For example, split thickness autographs generally show limited tissue expansion, require repeated surgical operations, and give rise to unfavorable aesthetic results. Epidermal autographs require long periods of time to be cultured, have a low success ("take") rate of approximately 30-48%, frequently form spontaneous blisters, exhibit contraction to 60-70% of their original size, are vulnerable during the first 15 days of engraftment, and are of no use in situations where there is both epidermal and dermal tissue involvement. Similarly, epidermal allografts (cultured allogenic keratinocytes) exhibit many of the limitations which are inherent in the use of epidermal autographs. Additional methodologies have been examined which involve the utilization of irradiated cadaver dermis. However, this too has met with limited success due to, for example, graft rejection and unfavorable aesthetic results.

Living skin equivalents comprising a dermal layer of *rodent* fibroblast cells cast in soluble collagen and an epidermal layer of cultured *rodent* keratinocytes have been successfully grafted as allografts onto Sprague Dawley rats by

Bell *et al.*, J. Investigative Dermatology 81:2 (1983). Histological examination of the engrafted tissue revealed that the epidermal layer had fully differentiated to form desmonosomes, tonofilaments, keratohyalin, and a basement lamella. However, subsequent attempts to reproduce the living skin equivalent using human fibroblasts and keratinocytes has met with only limited success. In general, the keratinocytes failed to fully differentiate to form a basement lamella and the dermo-epidermal junction was a straight line.

The present invention includes the following methodologies for the repair and/or augmentation of various skin defects: (1) the injection of autologously cultured dermal or fascial fibroblasts into various layers of the skin or injection directly into a "pocket" created in the region to be repaired or augmented, or (2) the surgical engraftment of "strands" derived from autologous dermal and fascial fibroblasts which are cultured in such a manner as to form a three-dimensional "tissue-like" structure similar to that which is found *in vivo*. Moreover, the present invention also differs on a two-dimensional level in that "true" autologous culture and preparation of the cells is performed by utilization of the patient's own cells and serum for *in vitro* culture.

III. VOCAL CORD TISSUE AUGMENTATION AND/OR REPAIR

Phonation is accomplished in humans by the passage of air past a pair of vocal cords located within the larynx. Striated muscle fibers within the larynx, comprising the constrictor muscles, function so as to vary the degree of tension in the vocal cords, thus regulating both their overall rigidity and proximity to one another to produce speech. However, when one (or both) of the vocal cords becomes totally or partially immobile, there is a diminution in the voice quality due to an inability to regulate and maintain the requisite tension and proximity of the damaged cord in

relation to that of the operable cord. Vocal cord paralysis may be caused by cancer, surgical or mechanical trauma, or similar afflictions which render the vocal cord incapable of being properly tensioned by the constrictor muscles.

One therapeutic approach which has been examined to allow phonation involves the implantation or injection of biocompatible materials. It has long been recognized that a paralyzed or damaged vocal cord may be repositioned or supported so as to remain in a fixed location relative to the operable cord such that the unilateral vibration of the operable cord produces an acceptable voice pattern. Hence, various surgical methodologies have been developed which involve the formation of an opening in the thyroid cartilage and subsequently providing a means for the support and/or repositioning of the paralyzed vocal cord.

For example, injection of TEFLON® into the paralyzed vocal cord to increase its inherent "bulk" has been described. See e.g., von Leden *et al.*, Phonosurgery 3:175 (1989). However, this procedure is now considered unacceptable due to the inability of the injected TEFLON® to close large glottic gaps, as well as its tendency to induce inflammatory reactions resulting in the formation of fibrous infiltration into the injected cord. See e.g., Maves et al., Phonosurgery: Indications and Pitfalls 98:577 (1989). Moreover, removal of the injected TEFLON® may be quite difficult should it subsequently be desired or become necessary.

Another methodology for supporting the paralyzed vocal cord which has been employed involves the utilization of a custom-fitted block of siliconized rubber (SILASTIC®). In order to ensure the proper fit of the implant, the surgeon hand carves the SILASTIC® block during the procedure in order to maximize the ability of the patient to phonate The patient is kept under local anesthesia so that he or she can produce sounds to test the positioning of the implant. Generally, the implanted blocks are formed into the shape of a wedge which is

totally implanted within the thyroid cartilage or a flanged plug which can be moved back-and-forth within the opening in the thyroid cartilage to fine-tune the voice of the patient.

Although SILASTIC® implants have proved to be superior over TEFLON® injections, there are several areas of dissatisfaction with the procedure including difficulty in the carving and insertion of the block, the large amount of time required for the procedure, and a lack of an efficient methodology for locking the block in place within the thyroid cartilage. In addition, vocal cord edema, due to the prolonged nature of the procedure and repeated voice testing during the operation, may also prove problematic in obtaining optimal voice quality.

Other methodologies which have been utilized in the treatment of vocal cord paralysis and damage include GELFOAM® hydroxyapatite, and porous ceramic implants, as well as injections of silicone and collagen. See e.g., Koufman, Laryngoplastic Phonosurgery (1988). However, these materials have also proved to be less than ideal due to difficulties in the sizing and shaping of the solid implants as well as the potential for subsequent immunogenic reactions. Therefore, there still remains a need for the development of a methodology which allows the efficacious treatment of vocal cord paralysis and/or damage.

SUMMARY OF THE INVENTION

The present invention discloses a methodology for the long-term augmentation and/or repair of dermal, subcutaneous, or vocal cord tissue by the injection or direct surgical placement/implantation of: (1) autologous cultured fibroblasts derived from connective tissue, dermis, or fascia; (2) lamina propria tissue; (3) fibroblasts derived from the lamina propria; or (4) adipocytes. The fibroblast cultures utilized for the augmentation and/or repair of skin defects are derived from either connective tissue, dermal, and/or fascial

fibroblasts. Typical defects of the skin which can be corrected with the injection or direct surgical placement of autologous fibroblasts or adipocytes include rhytids, stretch marks, depressed scars, cutaneous depressions of traumatic or non-traumatic origin, hypoplasia of the lip, and/or scarring from acne vulgaris. Typical defects of the vocal cord which can be corrected by the injection or direct surgical placement of lamina propria or autologous cultured fibroblasts from lamina propria include scarred, paralyzed, surgically or traumatically injured, or congenitally underdeveloped vocal cord(s).

The use of autologous cultured fibroblasts derived from the dermis, fascia, connective tissue, or lamina propria mitigates the possibility of an immunogenic reaction due to a lack of tissue histocompatibility. This provides vastly superior post-surgical results. In a preferred embodiment of the present invention, fibroblasts of connective tissue, dermal, or facial origin as well as adipocytes are derived from full-thickness biopsies of the skin. Similarly, lamina propria tissue or fibroblasts derived from the lamina propria are obtained from vocal cord biopsies. It should be noted that the aforementioned tissues are derived from the individual who will subsequently undergo the surgical procedure, thus mitigating the potential for an immunogenic reaction. These tissues are then expanded *in vitro* utilizing standard tissue culture methodologies.

Additionally, the present invention further provides a methodology of rendering the cultured cells substantially free of potentially immunogenic serum-derived proteins by late-stage passage of the cultured fibroblasts, lamina propria tissue, or adipocytes in serum-free medium or in the patient's own serum. In addition, immunogenic proteins may be markedly reduced or eliminated by repeated washing in phosphate-buffered saline (PBS) or similar physiologically-compatible buffers.

DESCRIPTION OF THE INVENTION

I. HISTOLOGY OF THE SKIN

The skin is composed of two distinct layers: the *epidermis,* a specialized epithelium derived from the ectoderm, and beneath this, the *dermis,* of vascular dense connective tissue, a derivative of mesoderm. These two layers are firmly adherent to one another and form a region which varies in overall thickness from approximately 0.5 to 4 mm in different areas of the body. Beneath the dermis is a layer of loose connective tissue which varies from areolar to adipose in character. This is the *superficial fascia* of gross anatomy, and is sometimes referred to as the *hypodermis,* but is not considered to be part of the skin. The dermis is connected to the hypodermis by connective tissue fibers which pass from one layer to the other.

A. EPIDERMIS

The epidermis, a stratified squamous epithelium, is composed of cells of two separate and distinct origins. The majority of the epithelium, of ectodermal origin, undergoes a process of keratinization resulting in the formation of the dead superficial layers of skin. The second component comprises the melanocytes which are involved in the synthesis of pigmentation via melanin. The latter cells do not undergo the process of keratinization. The superficial keratanized cells are continuously lost from the surface and must be replaced by cells that arise from the mitotic activity of cells of the basal layers of the epidermis. Cells which result from this proliferation are displaced to higher levels, and as they move upward they elaborate keratin, which eventually replaces the majority of the cytoplasm. As the process of keratinization continues the cell dies and is finally shed. Therefore, it should be appreciated that the structural organization of the epidermis into layers reflects

various stages in the dynamic process of cellular proliferation and differentiation.

B. DERMIS

It is frequently difficult to quantitatively differentiate the limits of the dermis as it merges into the underlying subcutaneous layer (hypodermis). The average thickness of the dermis varies from 0.5 to ≥ 3 mm and is further subdivided into two strata - the *papillary layer* superficially and the *reticular layer* beneath. The papillary layer is composed of thin collagenous, reticular, and elastic fibers arranged in an extensive network. Just beneath the epidermis, reticular fibers of the dermis form a close network into which the basal processes of the cells of the stratum germinativum are anchored. This region is referred to as the basal lamina.

The reticular layer is the main fibrous bed of the dermis. Generally, the papillary layer contains more cells and smaller and finer connective tissue fibers than the reticular layer. It consists of coarse, dense, and interlacing collagenous fibers, in which are intermingled a small number of reticular fibers and a large number of elastic fibers. The predominant arrangement of these fibers is parallel to the surface of the skin. The predominant cellular constituent of the dermis are fibroblasts and macrophages. In addition, adipose cells may be present either singly or, more frequently, in clusters. Owing to the direction of the fibers, lines of skin tension, *Langer's lines,* are formed. The overall direction of these lines is of surgical importance since incisions made parallel with the lines tend to gape less and heal with less scar tissue formation than incisions made at right-angles or obliquely across the lines. Pigmented, branched connective tissue cells, *chromatophores,* may also be present. These cells do not elaborate pigment but, instead, apparently obtain it from melanocytes.

Smooth muscle fibers may also be found in the dermis. These fibers are arranged in small bundles in connection with hair follicles (*arrectores pilorum* muscles) and are scattered throughout the dermis in considerable numbers in the skin of the nipple, penis, scrotum, and parts of the perineum. Contraction of the muscle fibers gives the skin of these regions a wrinkled appearance. In the face and neck, fibers of some skeletal muscles terminate in delicate elastic fiber networks of the dermis.

C. ADIPOSE TISSUE/ADIPOCYTES

Fat cells, or adipocytes, are scattered in areolar connective tissue. When adipocytes form large aggregates, and are the principle cell type, the tissue is designated adipose tissue. Adipocytes are fully differentiated cells and are thus incapable of undergoing mitotic division. New adipocytes therefore, which may develop at any time within the connective tissue, arise as a result of differentiation of more primitive cells. Although adipocytes, prior to the storage of lipid, resemble fibroblasts, it is likely that they arise directly from undifferentiated mesenchymal tissue.

Each adipocyte is surrounded by a web of fine reticular fibers; in the spaces between are found fibroblasts, lymphoid cells, eosinophils, and some mast cells. The closely spaced adipocytes form lobules, separated by fibrous septa. In addition, there is a rich network of capillaries in and between the lobules. The richness of the blood supply is indicative of the high rate of metabolic activity of adipose tissue.

It should be appreciated that adipose tissue is not static. There is a dynamic balance between lipid deposit and withdrawal. The lipid contained within adipocytes may be derived from three sources. Adipocytes, under the influence of the hormone insulin, can synthesize fat from carbohydrate. They can also produce fat from various fatty acids which are

derived from the initial breakdown of dietary fat. Fatty acids may also be synthesized from glucose in the liver and transported to adipocytes as serum lipoproteins. Fats derived from different sources also differ chemically. Dietary fats may be saturated or unsaturated, depending upon the individual diet. Fat which is synthesized from carbohydrate is generally saturated. Withdrawals of fat result from enzymatic hydrolysis of stored fat to release fatty acids into the blood stream. However, if there is a continuous supply of exogenous glucose, then fat hydrolysis is negligible. The normal homeostatic balance is affected by hormones, principally insulin, and by the autonomic nervous system, which is responsible for the mobilization of fat from adipose tissue.

Adipose tissue may develop almost anywhere areolar tissue is prevalent, but in humans the most common sites of adipose tissue accumulation are the subcutaneous tissues (where it is referred to as *the panniculus adiposus)*, in the mesenteries and omenta, in the bone marrow, and surrounding the kidneys. In addition to its primary function of storage and metabolism of neutral fat, in the subcutaneous tissue, adipose tissue also acts as a shock absorber and insulator to prevent excessive heat loss or gain through the skin.

II. HISTOLOGY OF THE LARYNX AND VOCAL CORDS

The larynx is that part of the respiratory system which connects the pharynx and trachea. In addition to its function as part of the respiratory system, it plays an important role in phonation (speech). The wall of the larynx is composed of a "skeleton" of hyaline and elastic cartilages, collagenous connective tissue, striated muscle, and mucous glands. The major cartilages of the larynx (the thyroid, cricoid, and arytenoids) are hyaline, whereas the smaller cartilages (the corniculates, cuneiforms, and the tips of the arytenoids) are elastic, as is the cartilage of the epiglottis. The aforementioned cartilages, together with the hyoid bone, are

connected by three large, flat membranes: the thyrohyoid, the quadrates, and the cricovocal. These are composed of dense fibroconnective tissue in which many elastic fibers are present, particularly in the cricovocal membrane. The true and false vocal cords (vocal and vestibular ligaments) are, respectively, the free upper boarders of the cricovocal (cricothyroid) and the free lower boarders of the quadrate (aryepiglottic) membranes. Extending laterally on each side between the true and false cords are the sinus and saccule of the larynx, a small slit-like diverticulum. Behind the cricoid and arytenoid cartilages, the posterior wall of the pharynx is formed by the striated muscle of the pharyngeal constrictor muscles.

The epithelium of the mucous membrane of the larynx varies with location. For example, over the vocal folds, the lamina propria of the stratified squamous epithelium is extremely dense and firmly bound to the underlying connective tissue of the vocal ligament. While there is no true submucosa in the larynx, the lamina propria of the mucous membrane is thick and contains large numbers of elastic fibers.

III. METHODOLOGIES

A. *IN VITRO* CELL CULTURE OF FIBROBLASTS OR LAMINA PROPRIA

While the present invention may be practiced by utilizing any type of non-differentiated mesenchymal cell found in the skin which can be expanded in *in vitro* culture, fibroblasts derived from dermal, connective tissue, fascial, lamina proprial tissues, adipocytes, and/or extracellular tissues derived from the cells are utilized in a preferred embodiment due to their relative ease of isolation and *in vitro* expansion in tissue culture. In general, tissue culture techniques which are suitable for the propagation of non-differentiated mesenchymal cells may be used to expand the aforementioned

cells/tissue and practice the present invention as further discussed below. See e.g., Culture of Animal Cells: A Manual of Basic Techniques, Freshney, R.I. ed., (Alan R. Liss & Co., New York 1987); Animal Cell Culture: A Practical Approach, Freshney, R.I. ed., (IRL Press, Oxford, England 1986), whose references are incorporated herein by reference.

The utilization of autologous engraftment is a preferred therapeutic methodology due to the potential for graft rejection associated with the use of allograft-based engraftment. Autologous grafts (i.e., those derived directly from the patient) ensure histocompatibility by initially obtaining a tissue sample via biopsy directly from the patient who will be undergoing the corrective surgical procedure and then subsequently culturing fibroblasts derived from the dermal, connective tissue, fascial, or lamina proprial regions contained therein.

While the following sections will primarily discuss the autologous culture of fibroblasts of connective tissue, dermal, or fascial origins, *in vitro* culture of lamina propria tissue may also be established utilizing analogous methodologies. An autologous fibroblast culture is preferably initiated by the following methodology. A full-thickness biopsy of the skin (~3x6 mm) is initially obtained through, for example, a punch biopsy procedure. The specimen is repeatedly washed with antibiotic and anti-fungal agents prior to culture. Through a process of sterile microscopic dissection, the keratinized tissue-containing epidermis and subcutaneous adipocyte-containing tissue is removed, thus ensuring that the resultant culture is substantially free of non-fibroblast cells (e.g., adipocytes and keratinocytes). The isolated adipocytes-containing tissue may then be utilized to establish adipocyte cultures. Alternately, whole tissue may be cultured and fibroblast-specific growth medium may be utilized to "select" for these cells.

Two methodologies are generally utilized for the autologous culture of fibroblasts in the practice of the present invention -- mechanical and enzymatic. In the mechanical methodology, the fascia, dermis, or connective tissue is initially dissected out and finely divided with scalpel or scissors. The finely minced pieces of the tissue are initially placed in 1-2 ml of medium in either a 5 mm petri dish (Costar), a 24 multi-well culture plate (Corning), or other appropriate tissue culture vessel. Incubation is preferably performed at 37°C in a 5% CO_2 atmosphere and the cells are incubated until a confluent monolayer of fibroblasts has been obtained. This may require up to 3 weeks of incubation. Following the establishment of confluence, the monolayer is trypsinized to release the adherent fibroblasts from the walls of the culture vessel. The suspended cells are collected by centrifugation, washed in phosphate-buffered saline, and resuspended in culture medium and placed into larger culture vessels containing the appropriate complete growth medium.

In a preferred embodiment of the enzymatic culture methodology, pieces of the finely minced tissue are digested with a protease for varying periods of time. The enzymatic concentration and incubation time are variable depending upon the individual tissue source, and the initial isolation of the fibroblasts from the tissue as well as the degree of subsequent outgrowth of the cultured cells are highly dependent upon these two factors. Effective proteases include, but are not limited to, trypsin, chymotrypsin, papain, chymopapain, and similar proteolytic enzymes. Preferably, the tissue is incubated with 200-1000 U/ml of collagenase type II for a time period ranging from 30 minutes to 24 hours, as collagenase type II was found to be highly efficacious in providing a high yield of viable fibroblasts. Following enzymatic digestion, the cells are collected by

centrifugation and resuspended into fresh medium in culture flasks.

Various media may be used for the initial establishment of an *in vitro* culture of human fibroblasts. Dulbecco's Modified Eagle Medium (DMEM, Gibco/BRL Laboratories) with concentrations of fetal bovine serum (FBS), cosmic calf serum (CCS), or the patient's own serum varying from 5-20% (v/v) -- with higher concentrations resulting in faster culture growth -- are readily utilized for fibroblast culture. It should be noted that substantial reductions in the concentration of serum (i.e., 0.5% v/v) results in a loss of cell viability in culture. In addition, the complete culture medium typically contains L-glutamine, sodium bicarbonate, pyridoxine hydrochloride, 1g/liter glucose, and gentamycin sulfate. The use of the patient's own serum mitigates the possibility of subsequent immunogenic reaction due to the presence of constituent antigenic proteins in the other serums.

Establishment of a fibroblast cell line from an initial human biopsy specimen generally requires 2 to 3.5 weeks in total. Once the initial culture has reached confluence, the cells may be passaged into new culture flasks following trypsinization by standard methodologies known within the relevant field. Preferably, for expansion, cultures are "split" 1:3 or 1:4 into T-150 culture flasks (Corning) yielding ~$5x10^7$ cells/culture vessel. The capacity of the T-150 culture flask is typically reached following 5-8 days of culture at which time the cultured cells are found to be confluent.

Cells are preferably removed for freezing and long-term storage during the early passage stages of culture, rather than the later stages due to the fact that human fibroblasts are capable of undergoing a finite numbers of passages. Culture medium containing 70% DMEM growth medium, 10% (v/v) serum, and 20% (v/v) tissue culture grade dimethylsulfoxide (DMSO, Gibco/BRL) may be effectively utilized for freezing of

fibroblast cultures. Frozen cells can subsequently be used to inoculate secondary cultures to obtain additional fibroblasts for use in the original patient, thus doing away with the requirement to obtain a second biopsy specimen.

To minimize the possibility of subsequent immunogenic reactions in the engraftment patient, the removal of the various antigenic constituent proteins contained within the serum may be facilitated by collection of the fibroblasts by centrifugation, washing the cells repeatedly in phosphate-buffered saline (PBS), and then either re-suspending or culturing the washed fibroblasts for a period of 2-24 hours in serum-free medium containing requisite growth factors which are well known in the field. Culture media include, but are not limited to, Fibroblast Basal Medium (FBM). Alternately, the fibroblasts may be cultured utilizing the patient's own serum in the appropriate growth medium.

After the culture has reached a state of confluence, the fibroblasts may either be processed for injection or further cultured to facilitate the formation of a three-dimensional "tissue" for subsequent surgical engraftment. Fibroblasts utilized for injection consist of cells suspended in a collagen gel matrix. The collagen gel matrix is preferably comprised of a mixture of 2 ml of a collagen solution containing 0.5 to 1.5 mg/ml collagen in 0.05% acetic acid, 1 ml of DMEM medium, 270 µl of 7.5% sodium bicarbonate, 48 µl of 100 µg/ml solution of gentamycin sulfate, and up to 5×10^6 fibroblast cells/ml of collagen gel. Following the suspension of the fibroblasts in the collagen gel matrix, the suspension is allowed to solidify for approximately 15 minutes at room temperature or 37°C in a 5% CO_2 atmosphere. The collagen may be derived from human or bovine sources, or from the patient and may be enzymatically- or chemically-modified (e.g., atelocollagen).

Three-dimensional "tissue" is formed by initially suspending the fibroblasts in the collagen gel matrix as

described above. Preferably, in the culture of three-dimensional tissue, full-length collagen is utilized, rather than truncated or modified collagen derivatives. The resulting suspension is then placed into a proprietary "transwell" culture system which is typically comprised of culture well in which the lower growth medium is separated from the upper region of the culture well by a microporous membrane. The microporous membrane typically possesses a pore size ranging from 0.4 to 8 μm in diameter and is constructed from materials including, but not limited to, polyester, nylon, nitrocellulose, cellulose acetate, polyacrylamide, cross-linked dextrose, agarose, or other similar materials. The culture well component of the transwell culture system may be fabricated in any desired shape or size (e.g., square, round, ellipsoidal, etc.) to facilitate subsequent surgical tissue engraftment and typically holds a volume of culture medium ranging from 200 μl to 5 ml. In general, a concentration ranging from 0.5×10^6 to 10×10^6 cells/ml, and preferably 5×10^6 cells/ml, are inoculated into the collagen/fibroblast-containing suspension as described above. Utilizing a preferred concentration of cells (i.e., 5×10^6 cells/ml), a total of approximately 4-5 weeks is required for the formation of a three-dimensional tissue matrix. However, this time may vary with increasing or decreasing concentrations of inoculated cells. Accordingly, the higher the concentration of cells utilized the less time due to a higher overall rate of cell proliferation and replacement of the exogenous collagen with endogenous collagen and other constituent materials which form the extracellular matrix synthesized by the cultured fibroblasts. Constituent materials which form the extracellular matrix include, but are not limited to, collagen, elastin, fibrin, fibrinogen, proteases, fibronectin, laminin, fibrellins, and other similar proteins. It should be noted that the potential for immunogenic reaction in the engrafted patient is markedly

reduced due to the fact that the exogenous collagen used in establishing the initial collagen/fibroblast-containing suspension is gradually replaced during subsequent culture by endogenous collagen and extracellular matrix materials synthesized by the fibroblasts.

B. *IN VITRO* CULTURE OF ADIPOCYTES

Adipocytes require a "feeder-layer" or other type of solid support on which to grow. One potential solid support may be provided by utilization of the previously discussed collagen gel matrix. Alternately, the solid support may be provided by cultured extracellular matrix. In general, the *in vitro* culture of adipocytes is performed by the mechanical or enzymatic disaggregation of the adipocytes from adipose tissue derived from a biopsy specimen. The adipocytes are "seeded" onto the surface of the aforementioned solid support and allowed to grow until near-confluence is reached. The adipocytes are removed by gentle scraping of the solid surface. The isolated adipocytes are then cultured in the same manner as utilized for fibroblasts as previously discussed in Section III A.

C. ISOLATION OF THE EXTRACELLULAR MATRIX

The extracellular matrix (ECM) may be isolated in either a cellular or acellular form. Constituent materials which form the ECM include, but are not limited to, collagen, elastin, fibrin, fibrinogen, proteases, fibronectin, laminin, fibrellins, and other similar proteins. ECM is typically isolated by the initial culture of cells derived from skin or vocal cord biopsy specimens as previously described. After the cultured cells have reached a minimum of 25-50% sub-confluence, the ECM may be obtained by mechanical, enzymatic, chemical, or denaturant treatment. Mechanical collection is performed by scraping the ECM off of the plastic culture vessel and re-suspending in phosphate-buffered saline

(PBS). If desired, the constituent cells are lysed or ruptured by incubation in hypotonic saline containing 5 mM EDTA. Preferably, however, scraping followed by PBS re-suspension is generally utilized. Enzymatic treatment involves brief incubation with a proteolytic enzyme such as trypsin. Additionally, the use of detergents such as sodium dodesyl sulfate (SDS) or treatment with denaturants such as urea or dithiotheritol (DTT) followed by dialysis against PBS, will also facilitate the release of the ECM from surrounding associated tissue.

The isolated ECM may then be utilized as a "filler" material in the various augmentation or repair procedures disclosed in the present application. In addition, the ECM may possess certain cell growth- or metabolism-promoting characteristics.

D. *IN VITRO* CULTURE OF FETAL OR JUVENILE CELLS OR TISSUES

In another preferred embodiment, rather than utilizing the patient's own tissue, all of the aforementioned cells, cell suspensions, or tissues may be derived from fetal or juvenile sources. Fetal cells lack the immunogenic determinants responsible for eliciting the host graft-rejection reaction and thus may be utilized for engraftment procedures with little or no probability of a subsequent immunogenic reaction. An acellular ECM may also be obtained from fetal ECM by hypotonic lysing of the constituent cells. The acellular ECM derived from fetal or juvenile sources or from *in vitro* culture of early passage cells typically possesses differs in both quantity and characteristics from that of the ECM derived from senescent or late-passage cells. The cellular or acellular ECM derived from fetal or juvenile sources may be used as a "filler" material in the various augmentation or repair procedures disclosed in the present application. In addition, the fetal

or juvenile ECM may possess certain cell growth- or metabolism-promoting characteristics.

E. INJECTION OF AUTOLOGOUS CULTURED DERMAL/FASCIAL FIBROBLASTS

To augment or repair dermal detects, autologously cultured fibroblasts are injected initially into the lower dermis, next in the upper and middle dermis, and finally in the subcutaneous regions of the skin as to form raised areas or "wheals." The fibroblast suspension is injected via a syringe with a needle ranging frog 30 to 18 gauge, with the gauge of the needle being dependent upon such factors as the overall viscosity of the fibroblast suspension and the type of anesthetic utilized. Preferably, needles ranging from 22 to 18 gauge and 30 to 27 gauge are used with general and local anesthesia, respectively.

To inject the fibroblast suspension into the lower dermis, the needle is placed at approximately a 45° angle to the skin with the bevel of the needle directed downward. To place the fibroblast suspension into the middle dermis the needle is placed at approximately a 20-30° angle. To place the suspension into the upper dermis, the needle is placed almost horizontally (i.e., ~10-15° angle). Subcutaneous injection is accomplished by initial placement of the needle into the subcutaneous tissue and injection of the fibroblast suspension during subsequent needle withdrawal. In addition, it should be noted that the needle is preferably inserted into the skin from various directions such that the needle tract will be somewhat different with each subsequent injection. This technique facilitates a greater amount of total skin area receiving the injected fibroblast suspension.

Following the aforementioned injections, the skin should be expanded and possess a relatively taut feel. Care should be taken so as not to produce an overly hard feel to the injected region. Preferably, depressions or rhytids appear

elevated following injection and 26 should be "overcorrected" by a slight degree of over-injection of the fibroblast suspension, as typically some degree of settling or shrinkage will occur post-operatively.

In some scenarios, the injections may pass into deeper tissue layers. For example, in the case of lip augmentation or repair, a preferred manner of injection is accomplished by initially injecting the fibroblast suspension into the dermal and subcutaneous layers as previously described, into the skin above the lips at the vermillion border. In addition, the vertical philtrum may also be injected. The suspension is subsequently injected into the deeper tissues of the lip, including the muscle, in the manner described for subcutaneous injection.

F. SURGICAL PLACEMENT OF AUTOLOGOUSLY CULTURED DERMAL/FASCIAL FIBROBLAST STRANDS

In a preferred methodology utilized to augment or repair the skin and/or lips by the surgical placement of autologously cultured dermal and/or fascial fibroblast strands, a needle (the "passer needle") is selected which is larger in diameter and greater in length than the area to be repaired or augmented . The passer needle is then placed into the skin and threaded down the length of the area. Guide sutures are placed at both ends through the dermal or fascial fibroblast strand. One end of the guide suture is fixed to a Keith needle which is subsequently placed through the passer needle. The guide suture is brought out through the skin on the side furthest (distal point) from the initial entry point of the passer needle. The dermal or fascial fibroblast graft is then pulled into the passer needle and its position may be adjusted by pulling on the distal point guide suture or, alternately, the guide suture closest to the passer needle entry point. While the dermal or fascial strand is held in place by the distal point suture, the passer needle is pulled backward and

removed, thus resulting in the final placement of the graft following the final cutting of the remaining suture.

Generally, the fascial or dermal graft is placed into the subcutaneous layer of the skin. However, in some situations, it may be placed either more deeply or superficially.

If the area to be repaired or augmented is either smaller or larger than would be practical to fill with the aforementioned needle method, a subcutaneous "pocket" may be created with a myringotomy knife, scissors, or other similar instrument. A piece of dermis or fascia is then threaded into this area by use of guide sutures and passer needle, as described above.

G. INJECTION OF CELLS OR OTHER SUBSTANCES INTO THE VOCAL CORDS OR LARYNX

Generally, it is not possible to inject cellular matter or other substances directly into the vocal cord epithelium due to its extreme thinness. Accordingly, injections are usually made into the lamina propria layer or the muscle itself.

Generally, lamina propria tissue (finely minced if required for injection), fibroblasts derived from lamina propria tissue, or gelatinous substances are utilized for injection. The preferable methodology consists of injection directly into the space containing the lamina propria, specifically into Reinke's space. Injection is accomplished by use of laryngeal injection needles of the smallest possible gauge which will accommodate the injectate without the use of extraneous pressure during the actual injection process. This is a subjective process as to the overall "feel" and the use of too much pressure may irreparably damage the injected cells. The material is injected via a syringe with a needle ranging from 30 to 18 gauge, with the gauge of the needle being dependent upon such factors as the overall viscosity of the injectate and the type of anesthetic utilized.

Preferably, needles ranging from 22 to 18 gauge and 30 to 27 gauge are used with general and local anesthesia, respectively. If required, several injections may be performed along the length of the vocal cord.

To medialize a vocal cord with autologously cultured fascial or dermal fibroblasts, the materials are preferably injected directly into the tissue lateral or at the lateral edge of the vocal cord. The fibroblasts may be injected into scar, Reinke's space, or muscle, depending upon the specific vocal cord pathology. Preferably, it would be injected into the muscle.

The procedure may be performed under general, local, topical, monitored, or with no anesthesia, depending upon patient compliance and tolerance, the amount of injected material, and the type of injection performed.

If a greater degree of augmentation is required, a "pocket" may be created by needle dissection. Alternately, laryngeal microdisection, using knives and dissectors, may be performed. The desired material is then placed into the pocket with laryngeal forceps, or directly injected, depending upon the size of the pocket, the size of the graft material, the anesthesia, and the open access. If the pocket is left open after the procedure, it is preferably closed with sutures, adhesive, or a laser, depending upon the size and availability of these materials and the individual preferences of the surgeon.

While embodiments and applications of the present invention have been described in some detail by way of illustration and example for purposes of clarity and understanding, it would be apparent to those individuals whom are skilled within the relevant art that many additional modifications would be possible without departing from the inventive concepts contained herein.

WHAT IS CLAIMED: 29

1. A method for corrective surgery in a human subject of a defect being amenable to rectification by the augmentation of tissue subjacent to the defect comprising the steps of:

a) retrieving a plurality of viable cells from the subject, the cells being selected from the group consisiting of fascia fibroblasts, connective tissue fibroblasts, lamina propria fibroblasts and adipocytes;

b) culturing the viable cells *in vitro*; and

c) placing an effective volume of the *in vitro* cultured cells into a tissue of the subject the tissue being located in a position subjacent to the defect to be rectified.

2. The method of claim 1 wherein the *in vitro* cultured cells are placed in the tissue of the subject by a method selected from injection, engraftment, engraftment by threading and direct placement.

3. The method of claim 1 wherein the cultured cells are suspended in a collagen or modified collagen solution prior to injection.

4. The method of claim 1 wherein the cells are cultured in a collagen or modified collagen matrix.

5. The method of claim 4 wherein the cells and the matrix are placed in the tissue by injection.

6. The method of claim 4 wherein the cells and the matrix are placed in the tissue by engraftment.

7. The method of claim 1 wherein serum from the subject is used for *in vitro* culture of the cells.

8. The method of claim 1 wherein the *in vitro* cultured cells are washed in phosphate buffered saline before being placed into the tissue of the subject.

9. The method of claim 1 wherein the defect is a rhytid, stretch mark, depressed scar, cutaneous depression, hypoplasia of the lip, or scarring from acne vulgaris.

10. The method of claim 9 wherein the defect is a rhytid, stretch mark, wrinkle, depressed scar, cutaneous depression, hypoplasia of the lip, prominent nasolabial fold, prominent melolabial fold, scarring from acne vulgaris, or post-rhinoplasty irregularity.

11. The method of claim 1 wherein the *in vitro* cultured cells are placed by injection into:

a) the lower dermis;

b) the middle dermis;

c) the upper dermis; and

d) the subcutaneous region of the skin.

12. A substantially pure *in vitro* produced extracellular matrix composition obtained from the process comprising the steps of:

a) culturing cells *in vitro* in a culture vessel for a time sufficient for the cells to produce extracellular matrix;

b) separating the extracellular matrix produced by the cultured cells from the culture vessel;

c) collecting the extracellular matrix.

13. The substantially pure in vitro produced extracellular matrix composition of claim 12, wherein the cells are from human fetus or neonate origin.

14. A method for corrective surgery in a human subject of a defect being amenable to rectification by the augmentation of tissue subjacent to the defect comprising the steps of:

a) retrieving a plurality of viable cells from the subject;

b) culturing the cells *in vitro* in a culture vessel for a time sufficient for the cells to produce extracellular matrix;

c) separating the extracellular matrix produced by the cells from the culture vessel;

d) collecting the extracellular matrix; and

e) placing the collected extracellular matrix into a tissue subjacent to the defect.

15. The method of claim 14 wherein the extracellular matrix is exposed to a hypotonic solution prior to being placed into the tissue subjacent to the defect.

16. A method for corrective surgery in a human subject of a vocal cord defect being amenable to rectification by the augmentation of tissue subjacent to the defect comprising the steps of:

a) retrieving a plurality of viable cells from the subject;

b) culturing the viable cells *in vitro*; and

c) placing an effective volume of the *in vitro* cultured cells into a tissue of the subject the tissue being located in a position subjacent to the defect to be rectified.

17. The method of claim 16 wherein the autologous *in vitro* cultured cells are placed in a site of the vocal cord selected from the group comprising a scar, Reinke's space, a muscle of the vocal cord, and the lamina propria.

18. The method of claim 14 wherein the defect is present in the vocal cord of the subject.

19. A method for corrective surgery in a human subject of a defect being amenable to rectification by the augmentation of tissue subjacent to the defect comprising the steps of:

a) retrieving a plurality of viable cells from a human fetus or neonate;

b) culturing the viable cells *in vitro*; and

c) placing an effective volume of the *in vitro* cultured cells into a tissue of the subject the tissue being located in a position subjacent to the defect to be rectified.

20. The method of claim 19 wherein the *in vitro* cultured cells are placed in the tissue of the subject by a method selected from injection, engraftment, engraftment by threading and direct placement.

21. The method of claim 19 wherein the cultured cells are suspended in a collagen or modified collagen solution prior to injection.

22. The method of claim 19 wherein the cells are cultured in a collagen or modified collagen matrix.

23. The method of claim 22 wherein the cells and the matrix are placed in the tissue by injection.

24. The method of claim 22 wherein the cells and the matrix are placed in the tissue by engraftment.

25. The method of claim 19 wherein the defect is a rhytid, stretch mark, depressed scar, cutaneous depression, hypoplasia of the lip, or scarring from acne vulgaris.

26. The method of claim 25 wherein the defect is a rhytid, stretch mark, wrinkle, depressed scar, cutaneous depression, hypoplasia of the lip, prominent nasolabial fold, prominent melolabial fold, scarring from acne vulgaris, or post-rhinoplasty irregularity.

27. The method of claim 19 wherein the *in vitro* cultured cells are placed by injection into:

a) the lower dermis;

b) the middle dermis;

c) the upper dermis; and

d) the subcutaneous region of the skin.

28. A method for corrective surgery in a human subject of a defect being amenable to rectification by the augmentation of tissue subjacent to the defect comprising the steps of:

a) retrieving a plurality of viable cells from a human fetus or neonate;

b) culturing the cells *in vitro* in a culture vessel for a time sufficient for the cells to produce extracellular matrix;

c) separating the extracellular matrix produced by the cells from the culture vessel;

d) collecting the extracellular matrix; and

e) placing the collected extracellular matrix into a tissue subjacent to the defect.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03348

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61F 2/00; 2/02, 2/10, 9/04; 13/00

US CL :424/422, 423, 426, 486; 613/11, 15

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/422, 423, 426, 486; 613/11, 15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, USPAT, EPO, JPO

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 95/24873 A1 (GOLDSTEIN) 21 September 1995, see abstract.	12 --- 1-11 and 13-28
X --- Y	US 5,567,612 A (VACANTI et al.) 22 October 1996, especially col. 1, line 23 - col. 3, line 7, and claims.	12 --- 1-11 and 13-28
X --- Y	US 5,041,138 A (VACANTI et al.) 20 August 1991, especially col. 1, line 18 - col. 5, line 7, and claims.	12 --- 1-11 and 13-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

CHRISTOPHER TATE

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03348

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,336,616 A (LIVESEY et al.) 09 August 1994, especially col. 1, line 16 - col. 7, line 8, and claims.	12 and 13
Y		1-11 and 14-28
Y	US 5,258,028 A (ERSEK et al.) 02 November 1993, especially Figures 3 and 4, and col. 1, line 10 - col. 3, line 6.	1-28
Y,P	US 5,633,001 A (AGERUP) 27 May 1997, especially col. 1, line 5 - col. 3, line 31.	1-28